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HIV-1 transgenic rat CD4+ T cells develop decreased CD28 responsiveness and suboptimal Lck tyrosine dephosphorylation following activation

Anjana Yadav [a], Shibani Pati [a], Anhthu Nyugen [a], Oxana Barabitskaja [a], Prosanta Mondal [c], Michael Anderson [a], Robert C. Gallo [a], David L. Huso [b], William Reid [a,*]

[a] Division of Basic Science, Institute of Human Virology, University of Maryland, Rm #S616, 725, West Lombard Street, Baltimore, MD 21201, USA
[b] Division of Comparative Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA
[c] Department of Epidemiology and Prevention, Institute of Human Virology, University of Maryland, Baltimore, MD 21201, USA

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Abstract

Impaired CD4+ T cell responses, resulting in dysregulated T-helper 1 (Th1) effector and memory responses, are a common result of HIV-1 infection. These defects are often preceded by decreased expression and function of the α/β T cell receptor (TCR)–CD3 complex and of co-stimulatory molecules including CD28, resulting in altered T cell proliferation, cytokine secretion and cell survival. We have previously shown that HIV Tg rats have defective development of T cell effector function and generation of specific effector/memory T cell subsets. Here we identify abnormalities in activated HIV-1 Tg rat CD4+ T cells that include decreased pY505 dephosphorylation of Lck (required for Lck activation), decreased CD28 function, reduced expression of the anti-apoptotic molecule Bcl-xL, decreased secretion of the mitogenic lympokine interleukin-2 (IL-2) and increased activation induced apoptosis. These events likely lead to defects in antigen-specific signaling and may help explain the disruption of Th1 responses and the generation of specific effector/memory subsets in transgenic CD4+ T cells.

Keywords: HIV-1; Transgenic; Lck; CD28; Memory

Introduction

Chronic HIV infection is distinguished by the gradual loss of naive and memory phenotype CD4+ T cells. Direct killing was initially thought to be the only mechanism by which HIV-1 leads to acquired immunodeficiency syndrome (AIDS). Qualitatively, however, early HIV infection affects the immune system even before CD4+ T cell depletion occurs. T cells from early asymptomatic infected persons with normal numbers of circulating CD4+ T helper cells have an imbalanced ratio of naive to memory phenotype CD4+ T cells and decreased T helper activity (Miedema et al., 1988; Shearer et al., 1984, 1986; Terpstra et al., 1989). It has been recently shown that during acute HIV and SIV infections there is a rapid and selective loss of memory phenotype CD4+ T cells at mucosal sites and in peripheral blood, with only a modest decrease in peripheral CD4+ T cell numbers (Brenchley et al., 2004; Mattapallil et al., 2005; Veazey et al., 1998), which may account for some (but not all) of the dysregulation in immune responses during early HIV-1 infection. The mechanism underlying this destruction of existent Th1 memory/effector cells in acute HIV-1 infection is not clear.

Activation of naive T cells is one of the earliest events in the generation of effector and memory cells. Optimal activation of naive T cells requires at least two signaling events (Bluestone, 1995; Bretscher and Cohn, 1970). The first is mediated by T cell receptor (TCR) engagement with antigen and MHC molecules on antigen presenting cells (APCs) and initiates activation of Lck and other intracellular signaling components. The second, termed co-stimulation, is most

* Corresponding author. Fax: +1 410 706 4694.
E-mail address: reid@umbi.umd.edu (W. Reid).

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frequently mediated by the engagement of the CD28 molecule on the naive T cell with B7-1 or B7-2 on the APC. CD28 co-stimulation enhances cell cycle entry by causing further changes in the phosphorylation status of Lck and activation of a restricted set of intracellular signaling components. This in turn stimulates expression of interleukin-2(IL-2) and anti-apoptotic molecules (most prominently Bcl-xL), and facilitates the acquisition of effector and memory responses as well as the induction of CD8+ T cell effector responses (Boise et al., 1995; Michel and Acuto, 2002; Sharpe and Freeman, 2002). A lack of CD28 engagement on naive TCR-stimulated T cells can result in clonal anergy (Harding et al., 1992; Schwartz, 1990) and/or T cell apoptosis (Kerstan and Hunig, 2004).

The differentiation of naive T cells into memory T cells is a crucial step in the evolution of an immune response and subsequent memory formation. Memory formation depends upon clonal expansion of naive T cells following an initial encounter with antigen (primary response), the acquisition of effector function (secretion of cytokines), a contraction or "death phase" during which effector cell numbers are reduced and finally memory development. As a consequence of clonal expansion during the primary response, the frequency of antigen-specific precursors significantly increase; conditions that affect the primary immune response may thus affect the overall generation of specific CD4+ T cell memory (Ahmed and Gray, 1996; Kaech et al., 2002). CD4+ T lymphocytes play a central role in the immune containment of HIV. They contribute to HIV clearance by providing help for B cell responses and by maintaining effective CTL populations (Sun et al., 2005). CD4+ T lymphocytes from HIV-1-infected individuals are qualitatively dysfunctional and are skewed to an early central memory phenotype (Yue et al., 2004), show a poor ability to provide help and also manifest a dysregulation in cytokine production such as reduced production of IL-2 and or IFN-γ, all of which may be associated with poor clinical outcome (Boaz et al., 2002; Sieg et al., 2001; Sun et al., 2005).

We previously described an HIV-1 transgenic rat that contains a *gag/pol*-deleted provirus (Reid et al., 2001, 2004), expresses viral RNA and proteins in lymphocytes and monocytes and develops Th1 immune response defects. The spleens from these Tg rats manifest extensive depletion of lymphocytes within the T cell region and B cell follicular hyperplasia. CD4+ and CD8+ T cells have defects in IFN-γ production, a skewed early central memory phenotype and an increase in activation-induced T cell apoptosis. Here we show defects in CD4+ T cells from these rats, including decreased Lck(pY505) dephosphorylation, CD28 function, expression of the anti-apoptotic factor Bcl-xL and secretion of IL-2 as well as increased activation-induced apoptosis in CD4 T cells following anti-CD3 and CD28 stimulation, all of which would likely lead to defects in antigen-specific signaling, Th1 responses and the generation of specific CD4+ T cell effector/memory subsets.

Results

HIV-1 Tg rat CD4+ T cells express reduced levels of CD28 following activation

The abnormally large population of T cells expressing the naive phenotype and the low number expressing the effector/memory phenotype in mature rats suggested that the T cells could be receiving suboptimal antigen stimulation; this led us to ask whether there was a correlation with defective activation and/or co-stimulation. We compared expression of CD28 following anti-CD3 stimulation of CD4+ T cells from PBMCs of mature (12–15 month old) Tg and control rats (n = 3) as judged by mean channel fluorescence intensity (MCF) measured by flow cytometry as described in Materials and methods. Fig. 1 shows that after stimulation, CD28 MCF of CD4+ T cells from the Tg rats was significantly decreased (130 ± 20 at 72 h) compared to age-matched controls (390 ± 124, $P < 0.05$), suggesting that CD28-mediated co-stimulation of naive CD4+ T cells might be inadequate.

Tg rat CD4+ T cells express reduced levels of IL-2 following activation

The reduced level of CD28 expressed by Tg rat CD4+ T cells following anti rat-CD3 stimulation led us to ask whether this could result in a reduced production of IL-2, a cytokine whose production requires CD28 signaling. Therefore, we compared production of IL-2 by purified CD4+ T cells from mature (12–15 months) Tg and control rats by intracellular flow cytometry and ELISA following stimulation. CD4+ T cells from mature rats were analyzed for intracellular IL-2 by flow cytometry as described in Materials and methods. In a representative experiment, shown in Figs. 2A and B, stimulation of CD4+ T cells led to an increase in the percentage of cells expressing IL-2, 11.7% in the non-Tg controls, compared to 2.3% in the Tg cells. The depicted percentages (panels A and B) are relative to isotype controls. Fig. 2C shows averages of the results from multiple experiments. A mean of 4.5 ± 2.0% Tg rat CD4+ (n = 5) T cells expressed

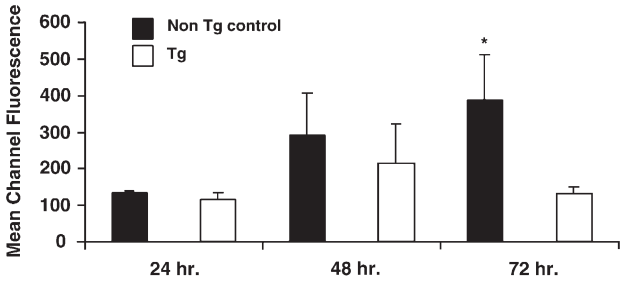

Fig. 1. Tg rat CD4 T cells express reduced surface CD28. Expression of CD28 (as a function of mean channel fluorescence) from activated CD4 T cells from 12–18 month old Tg (n = 3) and non-Tg rats (n = 3) was analyzed by flow cytometry. Times after stimulation at which analyses were performed are indicated. Error bars indicate standard deviations. * indicates a significant difference.

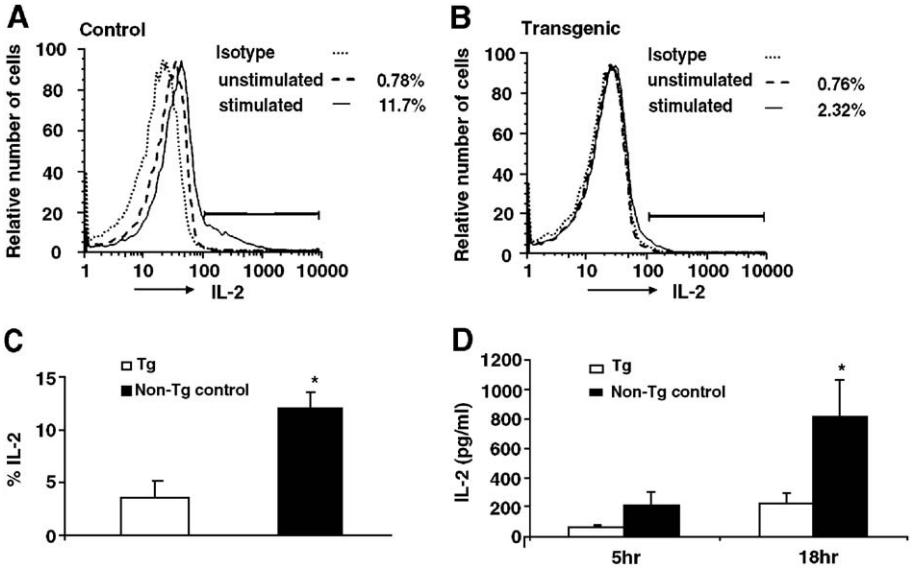

Fig. 2. Tg rat CD4 T cells express reduced levels of IL-2. Intracellular IL-2 production in peripheral blood T cells from Tg and non-Tg control rats is shown. Purified CD4+ T cells from 12–15 month old Fisher 344 Tg and non-Tg control rats were analyzed for intracellular IL-2 by flow cytometry following PMA-I stimulation as described in Materials and methods. Panels A and B show a representative set of data depicting differences in percent cells expressing IL-2 for control and Tg rats, respectively. Panel C shows the average of data sets for IL-2 intracellular staining with purified CD4+ T cells from multiple ($n = 5$) Tg and non-Tg control rats as indicated. Differences between Tg vs. age matched non-Tg controls were statistically significant ($P < 0.003$). Panel D shows extracellular IL-2 production from anti-CD3/anti-CD28 activated CD4+ T cells from 12–15 month old Fisher 344 Tg ($n = 3$) and non-Tg rats ($n = 3$). Samples were assayed in triplicate by capture ELISA as described in Materials and methods. Differences were statistically significant ($P < 0.04$). The numbers represent the mean values ± the standard deviation. * indicates a significant difference.

IL-2 vs. 12 ± 3.0% non-Tg control CD4+ ($n = 5$; $P < 0.003$) T cells. On average, although there was no significant change in the MCF from the IL-2 positive Tg CD4 T cells following stimulation compared to non-stimulated T cells (545 vs. 527, respectively), there was, however, an approximate 2-fold increase in MCF from the IL-2 producing CD4 positive T cells from non-Tg control following stimulation compared to non-stimulated controls (549 vs. 293, respectively). These data suggest that IL-2 production in the Tg rat is quantitatively and possible qualitatively dysregulated. In a separate set of experiments, CD4+ T cells were stimulated with plate bound anti-rat CD3 and CD28 as described in Materials and methods and extracellular production of IL-2 was measured by ELISA. Fig. 2D shows that IL-2 production from Tg CD4+ T cells ($n = 3$) was significantly decreased at 18 h (63 ± 10.4 pg/ml at 5 h and 257.3 ± 67.7 pg/ml at 18 h) relative to that of the age-matched controls ($n = 3$; 211.6 ± 93.8 pg/ml at 5 h and 821.6 ± 241.7 pg/ml at 18 h; $P < 0.04$). The reduction in IL-2 expression combined with reduced levels of cell surface CD28 suggest that either decreased levels of CD28 expression by Tg CD4+ T cells and/or defective CD3 or CD28 signaling contribute to this phenotype.

Tg rat CD4+ T cells express reduced levels of Bcl-xL following activation

The reduced level of CD28 expressed by Tg rat CD4+ T cells following anti rat-CD3 stimulation led us to ask whether this was correlated with reduced production of Bcl-xL, an anti-apoptotic molecule whose expression depends upon CD28-mediated signal transduction. Therefore, we compared expression of Bcl-xL mRNA and protein by real-time quantitative PCR and by flow cytometry 24 h after stimulation of CD4+ T cells with 10 μg/ml of anti-rat CD3 as described in Materials and methods. Fig. 3A shows an average of the results from multiple experiments, which indicate that non-Tg control cells experienced a significant ~2.5-fold increase in Bcl-xL mRNA ($P < 0.03$). In contrast, cells from age-matched Tg rats showed a moderate decrease in Bcl-xL mRNA. Fig. 3B shows that non-Tg CD4+ T cells treated under the same conditions also showed a significant increase in Bcl-xL protein, as judged by MCF and determined by flow cytometry (176 ± 7; $P < 0.05$) vs. age-matched control Tg rats (119 ± 11), in agreement with the RNA data. These differences are consistent with the idea that there is a deficit in CD28-mediated signal transduction, either because of low CD28 expression or decreased signaling through the CD28 receptor on Tg rat CD4+ T cells.

HIV-1 Tg rat CD4+ T cells show decreased Lck tyrosine dephosphorylation following activation

Although CD28 is constitutively expressed on the majority of T cells, its expression normally increases after T cell activation (Linsley et al., 1993). Protein tyrosine phosphorylation and dephosphorylation are essential steps in the signal transduction cascade leading to T cell activation.

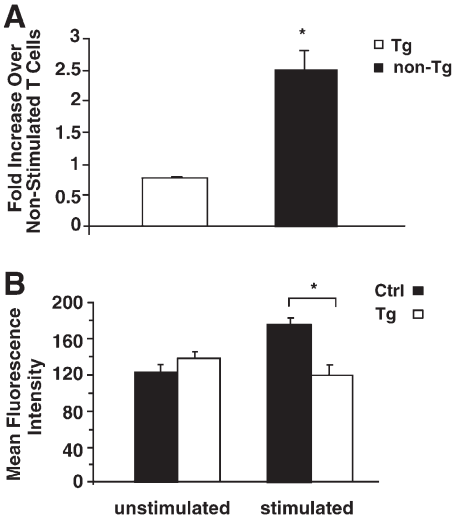

Fig. 3. Tg rat CD4+ T cells express reduced levels of Bcl-xL. PBMCs from Tg and non-Tg control Fisher 344 rats (n = 3) were stimulated with 10 μg/ml of anti rat CD3 for 24 h. Panel A shows expression of Bcl-xL mRNA by CD4+ T cells. CD4+ T cells for were purified by negative selection. Levels of Bcl-xL mRNA were determined by real-time quantitative PCR as described in Materials and methods. Samples were analyzed in triplicate and data were normalized to the expression of 18 s ribosomal mRNA. Differences were statistically significant ($P < 0.03$). The numbers represent the mean values ± the standard deviation. * indicates a significant difference. Panel B shows expression of Bcl-xL protein by CD4+ T cells. PBMC were stained for internal Bcl-xL protein and CD4 and mean channel fluorescence intensities were determined by flow cytometry as described in Materials and methods. Differences between stimulated non-Tg and Tg were statistically significant ($P < 0.05$). The numbers represent the mean values ± the standard deviation. * indicates a significant difference.

We wondered whether decreased TCR-mediated signal transduction could be related to the reduced levels of induction of CD28 on Tg rat CD4+ T cells following anti CD3 stimulation. Dephosphorylation of Lck at tyrosine residue 505 (Y505) is one of the early events following TCR stimulation. Hence, we determined the ability of non-Tg and Tg CD4+ T cells to dephosphorylate Lck (pY505) as an indicator of T cell activation. Purified CD4+ T cells were stimulated at indicated time points with cross-linked anti rat CD3–CD28 and the dephosphorylation of Lck at pY505 determined by Western blot analysis as described in Materials and methods. As indicated by the results from a representative experiment (Fig. 4), the ratio of pY505/total Lck following stimulation of the non-Tg control rat CD4+ T cells was approximately 4-fold decreased compared to that of Tg CD4+ T cells (0.57, 0.28 and 0.26 following incubation for 0, 0.5 and 1.5 min, respectively compared to ratios of 0.69, 0.51 and 1.2, respectively). Other Western blots gave generally similar results (not shown). We interpret this to mean that there is a deficit in Lck dephosphorylation that is related to a general dysregulation in TCR/CD28 signaling and may help to explain the decrease in IL-2 and Bcl-xL expression in activated CD4+ cells from the HIV-1 Tg rats.

HIV-1 Tg rats show increased activation-induced apoptosis of CD4+ lymphocytes following anti-CD3 activation

Failure to induce expression of Bcl-xL, an important anti-apoptotic molecule in activated T cells, could lead to elevated levels of activation-induced apoptosis. Apoptosis of CD4+ T cells in the HIV-1 Tg rat may help explain the abnormal disruption of Th1 responses and the dysregulated generation of specific effector/memory subsets. We have previously shown that mixed T cell populations from Tg rats are susceptible to induction of apoptosis following activation with PMA-I (Reid et al., 2004). Here we show that CD4+ T cells from Tg rats are more susceptible to apoptosis induction following stimulation than age-matched controls. PBMC were stimulated for 24 h with 10 μg/ml of plate bound mouse anti-rat CD3 as described in Materials and methods, and apoptosis was measured by staining CD3+CD4+ lymphocytes for surface exposure of Annexin V. Figs. 5A and B show representative Annexin V staining in stimulated and non-stimulated CD4+ T cells. Fig. 5A shows that 24 h following anti-CD3 stimulation, Annexin V labeling was 19% and 31% for non-Tg control and Tg rats, respectively. Fig. 5B shows that during the same time, Annexin V labeling for the non-stimulated CD4+ T cells was 8.2% and 7% for non-Tg controls and Tg rats, respectively. Fig. 5C shows that 24 h after stimulation, the mean percent change in Annexin V labeling in Tg CD4+ T cells was significantly greater than the controls (n = 6, 26.3 ± 9.9% vs. 14.6 ± 9.8%, $P < 0.03$). Unexpectedly, when PBMCs were co-stimulated with anti-CD3 plus anti-CD28 (10 μg/ml anti-CD3 and 1.5 μg/ml anti-CD28), there was an increase in apoptosis of the Tg CD4+ T cells, although it was not a significant increase. Apoptosis decreased in the controls, although again the differences between anti-CD3 alone and anti-CD3 plus anti-CD28 were also not significant. However, the difference between costimulated Tg and control CD4+ T cell apoptosis remained significant (n = 4, 30.0 ± 5.7% vs. 13.0 ± 6.7%, $P < 0.02$, respectively; data not shown). The cumulative data

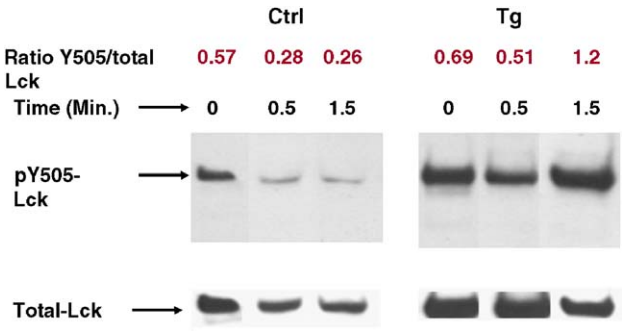

Fig. 4. Activation-induced Lck Tyrosine dephosphorylation of HIV-1 Tg rat CD4+ T cells is impaired. Purified CD4+ T (1.0×10^6/ml) cells were pre-incubated with (10 μg/ml) mouse anti-rat CD3 and CD28 for 30 min at 4 °C, washed and incubated with 10 μg/ml sheep anti-mouse IgG for 30 min at 4 °C. Cells were incubated for the indicated times at 37 °C before addition of RIPA lysis buffer. Proteins in the CD4+ T cell lysate were resolved by SDS-PAGE, Western blotted and probed with anti-rat phospho-specific Lck p56 (pY505; upper panel) stripped and probed with total Lck p56 (lower panel) as indicated.

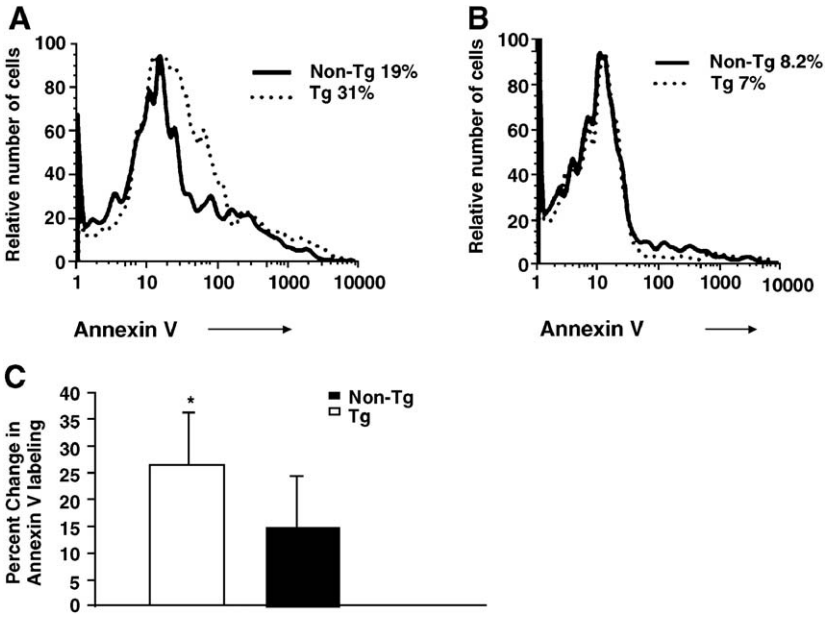

Fig. 5. Increased activation-induced apoptosis of HIV-1 Tg rat CD4+ lymphocytes following anti CD3 activation. Transgenic ($n = 6$) and age-matched controls ($n = 6$) PBMCs were stimulated with plate bound mouse anti-rat CD3 for 24 h at 37 °C. Apoptosis was determined as the difference from stimulated and non-stimulated in CD3+ CD4+ lymphocytes in the percent of Annexin V labeling as described in Materials and methods. Panels A and B show representative Annexin V staining for stimulated and non-stimulated populations, respectively. Panel C shows the percent changes in Annexin V labeling. The numbers represent the mean values ± the standard deviation. * indicates a significant difference ($P < 0.03$).

suggest that Tg rat CD4+ T cells show a qualitative and/or quantitative defect in CD3/CD28 signaling that may contribute to decreased Bcl-xL production, Lck activation, increased CD4+ T cell death and abnormal Th1 and memory responses and the apoptosis of these cells is not rescued by increasing CD28 signaling.

Discussion

We have previously reported that HIV-1 Tg rats have an increased susceptibility of CD3+ T cells to apoptosis following activation with PMA-I and impaired Th1 immunity, as evidenced by a reduced DTH reaction to antigen in vivo and IFN-γ production following activation in vitro. Consistent with the idea that there is a defect in the development of effector/memory cells, older but not young HIV-1 Tg rats have reduced numbers of CD4+ and CD8+ cells with an effector/memory surface phenotype and a reciprocal increase in the numbers of CD4+ and CD8+ cells with a naive surface phenotype (Reid et al., 2001, 2004). Here we show that activation of Tg rat CD4+ T cells with anti CD3 with or without anti CD28 is marked by reduced Lck (pY505) dephosphorylation, reduced induction of cell surface CD28, low expression of IL-2 and Bcl-xL, and increased activation induced apoptosis. These findings were evident in mature (12–15 months) rats of both Fisher 344 and Sprague–Dawley strains. It is likely that the inability to dephosphorylate Lck (pY505) is an early signaling event and leads to the other differences between Tg rats and age-matched controls. Inefficient Lck (pY505) dephosphorylation would preclude proper Lck activation, which in turn would hinder CD4+ T cell responses, including T cell antigen receptor (TCR)/CD28 signaling, Th1 cytokine production, effector/memory development and resistance to activation induced apoptosis.

These outlined immune abnormalities together with the large population of Tg rat CD4+ T cells expressing the naive phenotype and the reciprocal paucity of effector/memory phenotype CD4+ T cells, as previously reported (Reid et al., 2004), suggest that the reduced sizes in memory phenotype CD4+ T cells seen in the Tg rats result from suboptimal priming, dysregulated T cell expansion and/or differentiation or increased apoptosis. This idea that the Tg rats show a defect in T cell activation is supported by data from CD4 T cells of young Tg rats (with a normal distribution of naive and effector/memory phenotype T cell subsets) which demonstrate a reduced ability to proliferate following anti CD3/28 stimulation compared to age-matched controls (data not shown). Despite replicating some of the immunopathologies in humans, viral expression in many existent HIV-1 Tg mouse models is regulated either by heterologous promoters, which may not accurately reproduce the patterns of expression driven in natural infection by the viral promoter, or by viral promoter itself, which gives the highest expression in the skin (Dickie et al., 1991), likely because mouse cyclin T does not interact functionally with Tat (Wei et al., 1998). Although the Tg rat model that we have developed is also regulated by the viral promoter, the rat cyclin T appears to be functional (Bieniasz and Cullen, 2000; Keppler et al., 2001), resulting in efficient viral transcription in lymph nodes, spleen, thymus and blood (Reid et al., 2001, 2004). The Tg rats develop defects in Th1 immunity, their spleens manifest extensive depletion of lymphocytes within the T cell region and B cell follicular hyperplasia; Tg T cells are also marked by a skewed early central memory

phenotype, and an increase in activation-induced T cell apoptosis. Thus, it would be interesting to speculate, based upon these observations and the recently observed immune dysfunctions reported here, that during human HIV-1 infection, HIV-1 accessory and/or structural proteins may induce similar alterations in immune responses in affected CD4+ T cells, decreasing antigen-specific primary responses, central memory (T_{CM}) recall responses and/or the development of functional effectors before classical characteristics of AIDS develop.

Memory T cells are heterogeneous in terms of their homing capacity and effector function. This heterogeneity is reflected in the definition of T_{CM} and effector memory (T_{EM}) T cells (Lanzavecchia and Sallusto, 2005; Sallusto et al., 2004). T_{CM} home to lymph nodes, have limited effector function but can proliferate and become effector cells upon secondary stimulation while, T_{EM} home to peripheral tissues, rapidly produce effector cytokines but have a limited capacity to proliferate. During a primary immune response, the strength of TCR and cytokine stimulation received activate T cells to progressively acquire tissue homing receptors, effector function and the capacity to respond to homeostatic cytokines and appear as a heterogeneous continuum in terms of cell divisions, phenotype and function (Lanzavecchia and Sallusto, 2005). In CD4+ T cells, this response declines quickly after antigen withdrawal and a spectrum of resting memory cell subsets reflects the subset at the peak of antigenic response(Ahmed and Gray, 1996; Kaech et al., 2002; Lanzavecchia and Sallusto, 2005; Roman et al., 2002). During the generation of memory, T cells receiving weak stimulation are programmed to die because they express low levels of anti-apoptotic molecules such as Bcl-xL (Gett et al., 2003; Rathmell and Thompson, 2002). Reports also demonstrate that, in naive CD4+ T cells, CD28 can specifically enhance TCR signaling pathways; thus, CD28 signals provide a key quantitative contribution to potently boost the TCR signal under low occupancy (Acuto et al., 2003). In our study, it would be interesting to speculate that the reduced Lck activation in CD4+ T cells following TCR/CD28 receptor ligation (Fig. 4) results in qualitative and/or quantitative defects in T cell activation and CD28 co-stimulation, reduction in IL-2 production, Bcl-xL expression and increased susceptibility of Tg CD4+ T cells to activation-induced apoptosis, culminating in a dysregulation of memory T cell development, as previously reported in the HIV-1 Tg rats (Reid et al., 2004).

Which viral proteins are responsible for the observed abnormalities is of obvious interest. The *gag* and *pol* genes are deleted in the transgene in these rats, but all other viral genes are present, including the viral regulatory genes. Interestingly, the HIV-1 protein Nef has been reported to interact with specific cellular proteins involved in signal transduction (Greenway et al., 1996, 2003). Of particular interest, Nef has multiple independent effects on normal T cell function that include inhibition of signal transduction through the T cell receptor CD3 complex resulting in the down-regulation of the transcription factors NF-κB and AP1, inhibition of Lck activation, modulation of several other effector and signaling pathways in T cells such as PAK and PKC kinases, activation of NFAT1 and modulation of calcium flux. Nef also down-regulates CD4 and CD28 expression, inhibits induction of IL-2 mRNA and inhibits the expression of Bcl-xL and its anti-apoptotic effect following T cell activation (Bandres and Ratner, 1994; Greenway et al., 1996; Lu et al., 1996; Luria et al., 1991; Manninen et al., 2000; Smith et al., 1996; Tuosto et al., 2003; Keppler et al., 2005). Reduced CD4+ T cell activation/signaling could therefore result from Nef binding to Lck and inhibiting pY505 dephosphorylation/activation. Whether Nef is important in the HIV-1 Tg rat is not yet clear; however, many of the immune irregularities correlate with the above activities of the Nef protein. We are currently investigating this possibility in the Tg rat.

In summary, we describe CD4 T cell abnormalities in the HIV-1 Tg rat that include a dysregulation in Lck (pY505) dephosphorylation, reduced CD28, IL-2 and Bcl-xL expression and increased activation induced apoptosis. We hypothesize that the inability to dephosphorylate Lck (pY505) stalls Lck activation, which in turn hinders CD4+ T cell processes such as TCR/CD28 signaling, Th1 cytokine production and effector/memory development, while increasing their susceptibility to activation induced apoptosis. A subtle decreased cellularity of the thymic cortex, which has been noted histologically in the transgenic rats (Huso, unpublished), lends further support to the notion of altered Lck signaling since dysregulated p56lck signaling has been shown to affect multiple stages of thymocyte development and can result in a reduction in double positive cells in the thymic cortex of rodents(Molina et al., 1992). Although we do not yet have direct evidence for involvement of the HIV-1 protein Nef in T cell dysfunctions observed in the HIV-1 Tg rat, based on the reported functions of this protein, it is likely that Nef plays a role in the inhibition of key members of the TCR signaling cascade. Nef may alter the ability of the HIV-1 Tg rat CD4+ T cells to respond to antigens or cytokines and contribute to the dysregulated immune responses we describe here.

Materials and methods

HIV-1 and non-Tg animals

The construction of the HIV-1 transgene and production of the Tg rats have been described (Reid et al., 2001). Mature (12–15 months old) specific pathogen-free (SPF) Fisher 344/NHsd or Sprague–Dawley Tg rats and age-matched non-Tg rats were used in our analysis. These animals were housed under pathogen-free conditions in micro-isolator cages on HEPA filtered ventilated racks. The University of Maryland Biotechnology Institute Animal Care and Use Committee approved the experimental protocol.

Isolation of peripheral blood mononuclear and CD4+ T cells

Isolation of PBMCs has been described (Reid et al., 2004). CD4+ T cells were isolated by negative selection using the protocol described by the manufacturer (StemSep, Vancouver, BC, Canada). Briefly, PBMCs were resuspended at 5.0×10^7 cells/ml and 100 μl of rat CD4 antibody cocktail/ml was added

to cells for 30 min at 4 °C. Then, 60 μl of magnetic colloid/ml was added and incubated for 30 min at 4 °C. CD4+ T cells were isolated by magnetic separation. Purity of CD4+ T cells was generally >95%.

Analysis of IL-2, Bcl-xL and CD28 expression by flow cytometery

Expression of surface and intracellular proteins was assessed by four-color flow cytometric analysis. PBMCs or CD4 T cells (1.0×10^6/ml) from Tg and normal controls Fisher 344/NHsd were analyzed for IL-2 and Bcl-xL production by intracellular staining. Briefly, for IL-2 staining, purified CD4 T cells were cultured in RPMI media 1640 supplemented with 10% heat-inactivated fetal bovine serum (Valley Biomedical, Winchester, Va.), stimulated for 2 h with 25 ng/ml of phorbol 12-myristrate 13-acetate and 1 μg/ml of ionomycin (PMA-I), treated with GolgiStop (Beckton Dickerson (BD) Biosciences, San Jose, CA) and cultured in 5% CO_2 at 37 °C for an additional 4 h. For Bcl-xL staining, PBMCs were stimulated with 10 μg/ml plate bound purified anti-rat CD3 (G4.18, PharMingen) for 24 h. At the appropriate time, cells were stained with primary antibody or an appropriate isotype control according to the manufacturer's instructions. Purified CD4 T cells or PBMCs were stained with FITC anti-rat αβ T cell receptor (R73, PharMingen), and APC anti-rat CD4 (OX-35, PharMingen). Following surface staining, cells were fixed, permeabilized and stained with PE anti-rat IL-2 (BL-7015, BioLegend) or PE anti-human Bcl-xL (7B2.5, SouthernBiotech) according to the manufacturer's recommendations. For analysis of CD28 surface expression, PBMCs were stimulated with 10 μg/ml plate bound purified anti-rat CD3 (G4.18, PharMingen) for the indicated time and then stained with FITC anti-rat αβ T cell receptor (R73, PharMingen), PE anti-rat CD28 (JJ319, PharMingen) and APC anti-rat CD4 (OX-35, PharMingen). Samples were acquired using a FACSCalibur (BD Biosciences) flow cytometer, and the data analyzed by FlowJo software (Tree Star, Inc., San Carlos, CA.).

Analysis of IL-2 in culture supernatants

Purified CD4+ T cells (1.0×10^6/ml) from Tg and control Fisher rats were stimulated with 10 μg/ml of plate bound purified anti-rat CD3 (G4.18, PharMingen) and CD28 (JJ319, PharMingen), as described above and their supernatants collected at the indicated times. Media were collected and analyzed in triplicate. IL-2 levels were measured using an ELISA cytokine detection kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol.

Apoptosis assays

Apoptosis was assayed by surface staining for phosphotidylserine using Annexin V-PE (Pharmingen), and necrotic cells were stained with 7-AAD (Pharmingen), according to the manufacturer's instructions. PBMCs from Tg and non-Tg rats were suspended in RPMI 1640 supplemented with 10% heat inactivated FBS, and plated at 2×10^5 cells/well. The cells were stimulated with purified plate bound anti-CD3 (10 μg/ml, G4.18, PharMingen) for 24 h at 37 °C and 5% CO_2. Cells were then stained with FITC-αβ-TCR (R73, PharMingen), PE-Annexin-V, 7-AAD and APC-CD4 (OX-35) antibodies (PharMingen). Samples were acquired using FACSCalibur flow cytometer (BD Biosciences). Analysis was done using FlowJo software. Data are represented as differences in the percent of Annexin V binding between stimulated and non-stimulated CD4+ T cells.

Western blot analysis

Purified rat CD4+ T cells were stimulated as previously described (Dennehy et al., 2003). Briefly, purified CD4+ T cells were stimulated with crosslinked (anti-mouse IgG, M 9902, SIGMA), purified mouse anti-rat CD3 (G4.18, PharMingen) and CD28 (JJ319, PharMingen). Cells were harvested and lysed, at indicated times, in lysis buffer (5% Triton X100 in PBS, pH 7.4) with protease inhibitors (complete with mini-leupeptin, aprotinin and Pefabloc) (Boehringer-Mannheim). Protein was quantified by BCA assay (Pierce Biochemicals). Protein was denatured at 70 °C for 10 min in 1× LDS loading buffer (Invitrogen, Carlsbad CA). Equal amounts of protein were loaded in each well and electrophoresed on a 4–12% bis-Tris gel (NuPAGE) (Invitrogen) on the NOVEX X-Cell II system (Invitrogen, Carlsbad, CA). Transfer to nitrocellulose membranes (Optitran, Schleicher and Schuell, Keene NH) was performed in a NOVEX transfer system X-Cell II (Invitrogen). Transfer buffer contained 25 mM bicine, 25 mM bis-Tris, 1.025 mM EDTA, 0.05 mM chlorbutanol and 15% methanol. Blots were blocked in PBS-5% non-fat dried milk. Membranes were probed with primary antibodies to phospho-Lck (pY505, clone 4, BD Biosciences) and total Lck (3A5, Santa Cruz Biotechnology). Secondary antibody was conjugated with horseradish peroxidase (HRP) (Cell Signaling) and detected with an ECL detection system (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's protocol.

Real-time PCR for Bcl-xL

To measure the relative amount of defined mRNA, quantitative real-time PCR analysis was performed using an ABI GeneAmp 5700 Thermocycler Sequence Detection System (Applied Biosystems, Foster City, CA). Purified CD4+ T cells were isolated from control and Tg rats and stimulated at 1.0×10^6 cells/ml for 24 h with 10 μg/ml anti-rat CD3 (G4.18, PharMingen) and CD28 (JJ319, PharMingen). Total cellular RNA was prepared using the Trizol reagent (Invitrogen) and the volume of each sample was adjusted to 20 μl using DEPC-treated water. Synthesis of first-strand cDNA (RT reaction) was performed under standard conditions using 4 μl total cellular RNA, 2.5 μM random hexamer and 200 units of SuperScript II RT for 1 h at 42 °C, followed by 5 min at 99 °C. Quantitative real-time RT-PCR was carried out using QuantiTect SYBR

Green PCR kit (Qiagen, Valencia, CA). The rat Bcl-xL gene was amplified with the following primers: forward 5′-GTAC-CGGAGAGCATTCAGTG-3′ and reverse 5′-CCTGCATC-TCCTTGTCTACG-3′. Samples were run in triplicate and the yield of Bcl-xL PCR product was normalized to the yield of PCR product generated using an rRNA 18S primer set (Ambion, Austin, TX). As a DNA contamination control, equal amounts of RNA were used without reverse transcriptase.

Statistics

Mean expression for CD28, IL-2 and Bcl-xL was compared using an independent Student *t* test. The percent of apoptotic CD4+ T cells was compared within and between groups by the Mann–Whitney test for samples exhibiting non-normal distribution. *P* values were considered significant at $P < 0.05$.

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References

Acuto, O., Mise-Omata, S., Mangino, G., Michel, F., 2003. Molecular modifiers of T cell antigen receptor triggering threshold: the mechanism of CD28 costimulatory receptor. Immunol. Rev. 192, 21–31.

Ahmed, R., Gray, D., 1996. Immunological memory and protective immunity: understanding their relation. Science 272, 54–60.

Bandres, J.C., Ratner, L., 1994. Human immunodeficiency virus type 1 Nef protein down-regulates transcription factors NF-kappa B and AP-1 in human T cells in vitro after T-cell receptor stimulation. J. Virol. 68, 3243–3249.

Bieniasz, P.D., Cullen, B.R., 2000. Multiple blocks to human immunodeficiency virus type 1 replication in rodent cells. J. Virol. 74, 9868–9877.

Bluestone, J.A., 1995. New perspectives of CD28-B7-mediated T cell costimulation. Immunity 2, 555–559.

Boaz, M.J., Waters, A., Murad, S., Easterbrook, P.J., Vyakarnam, A., 2002. Presence of HIV-1 Gag-specific IFN-gamma+IL-2+ and CD28+IL-2+ CD4 T cell responses is associated with nonprogression in HIV-1 infection. J. Immunol. 169, 6376–6385.

Boise, L.H., Minn, A.J., Noel, P.J., June, C.H., Accavitti, M.A., Lindsten, T., Thompson, C.B., 1995. CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-XL. Immunity 3, 87–98.

Brenchley, J.M., Schacker, T.W., Ruff, L.E., Price, D.A., Taylor, J.H., Beilman, G.J., Nguyen, P.L., Khoruts, A., Larson, M., Haase, A.T., Douek, D.C., 2004. CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. J. Exp. Med. 200, 749–759.

Bretscher, P., Cohn, M., 1970. A theory of self-nonself discrimination. Science 169, 1042–1049.

Dennehy, K.M., Kerstan, A., Bischof, A., Park, J.H., Na, S.Y., Hunig, T., 2003. Mitogenic signals through CD28 activate the protein kinase Ctheta-NF-kappaB pathway in primary peripheral T cells. Int. Immunol. 15, 655–663.

Dickie, P., Felser, J., Eckhaus, M., Bryant, J., Silver, J., Marinos, N., Notkins, A.L., 1991. HIV-associated nephropathy in transgenic mice expressing HIV-1 genes. Virology 185, 109–119.

Gett, A.V., Sallusto, F., Lanzavecchia, A., Geginat, J., 2003. T cell fitness determined by signal strength. Nat. Immunol. 4, 355–360.

Greenway, A., Azad, A., Mills, J., McPhee, D., 1996. Human immunodeficiency virus type 1 Nef binds directly to Lck and mitogen-activated protein kinase, inhibiting kinase activity. J. Virol. 70, 6701–6708.

Greenway, A.L., Holloway, G., McPhee, D.A., Ellis, P., Cornall, A., Lidman, M., 2003. HIV-1 Nef control of cell signalling molecules: multiple strategies to promote virus replication. J. Biosci. 28, 323–335.

Harding, F.A., McArthur, J.G., Gross, J.A., Raulet, D.H., Allison, J.P., 1992. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. Nature 356, 607–609.

Kaech, S.M., Wherry, E.J., Ahmed, R., 2002. Effector and memory T-cell differentiation: implications for vaccine development. Nat. Rev., Immunol. 2, 251–262.

Keppler, O.T., Yonemoto, W., Welte, F.J., Patton, K.S., Iacovides, D., Atchison, R.E., Ngo, T., Hirschberg, D.L., Speck, R.F., Goldsmith, M.A., 2001. Susceptibility of rat-derived cells to replication by human immunodeficiency virus type 1. J. Virol. 75, 8063–8073.

Keppler, O.T., Allespach, I., Schuller, L., Fenard, D., Greene, W.C., Fackler, O.T., 2005. Rodent cells support key functions of the human immunodeficiency virus type 1 pathogenicity factor Nef. J. Virol. 79, 1655–1665.

Kerstan, A., Hunig, T., 2004. Cutting edge: distinct TCR- and CD28-derived signals regulate CD95L, Bcl-xL, and the survival of primary T cells. J. Immunol. 172, 1341–1345.

Lanzavecchia, A., Sallusto, F., 2005. Understanding the generation and function of memory T cell subsets. Curr. Opin. Immunol. 17, 326–332.

Linsley, P.S., Bradshaw, J., Urnes, M., Grosmaire, L., Ledbetter, J.A., 1993. CD28 engagement by B7/BB-1 induces transient down-regulation of CD28 synthesis and prolonged unresponsiveness to CD28 signaling. J. Immunol. 150, 3161–3169.

Lu, X., Wu, X., Plemenitas, A., Yu, H., Sawai, E.T., Abo, A., Peterlin, B.M., 1996. CDC42 and Rac1 are implicated in the activation of the Nef-associated kinase and replication of HIV-1. Curr. Biol. 6, 1677–1684.

Luria, S., Chambers, I., Berg, P., 1991. Expression of the type 1 human immunodeficiency virus Nef protein in T cells prevents antigen receptor-mediated induction of interleukin 2 mRNA. Proc. Natl. Acad. Sci. U.S.A. 88, 5326–5330.

Manninen, A., Renkema, G.H., Saksela, K., 2000. Synergistic activation of NFAT by HIV-1 nef and the Ras/MAPK pathway. J. Biol. Chem. 275, 16513–16517.

Mattapallil, J.J., Douek, D.C., Hill, B., Nishimura, Y., Martin, M., Roederer, M., 2005. Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. Nature 434, 1093–1097.

Michel, F., Acuto, O., 2002. CD28 costimulation: a source of Vav-1 for TCR signaling with the help of SLP-76? Sci. STKE E35.

Miedema, F., Petit, A.J., Terpstra, F.G., Schattenkerk, J.K., de Wolf, F., Al, B.J., Roos, M., Lange, J.M., Danner, S.A., Goudsmit, J., 1988. Immunological abnormalities in human immunodeficiency virus (HIV)-infected asymptomatic homosexual men. HIV affects the immune system before CD4+ T helper cell depletion occurs. J. Clin. Invest. 82, 1908–1914.

Molina, T.J., Kishihara, K., Siderovski, D.P., van Ewijk, W., Narendran, A., Timms, E., Wakeham, A., Paige, C.J., Hartmann, K.U., Veillette, A., 1992. Profound block in thymocyte development in mice lacking p56lck. Nature 357, 161–164.

Rathmell, J.C., Thompson, C.B., 2002. Pathways of apoptosis in lymphocyte development, homeostasis, and disease. Cell 109, S97–S107 (Suppl.).

Reid, W., Sadowska, M., Denaro, F., Rao, S., Foulke Jr., J., Hayes, N., Jones, O., Doodnauth, D., Davis, H., Sill, A., O'Driscoll, P., Huso, D., Fouts, T., Lewis, G., Hill, M., Kamin-Lewis, R., Wei, C., Ray, P., Gallo, R.C., Reitz, M., Bryant, J., 2001. An HIV-1 transgenic rat that develops HIV-related pathology and immunologic dysfunction. Proc. Natl. Acad. Sci. U.S.A. 98, 9271–9276.

Reid, W., Abdelwahab, S., Sadowska, M., Huso, D., Neal, A., Ahearn, A., Bryant, J., Gallo, R.C., Lewis, G.K., Reitz, M., 2004. HIV-1 transgenic rats develop T cell abnormalities. Virology 321, 111–119.

Roman, E., Miller, E., Harmsen, A., Wiley, J., von Andrian, U.H., Huston, G., Swain, S.L., 2002. CD4 effector T cell subsets in the response to influenza: heterogeneity, migration, and function. J. Exp. Med. 196, 957–968.

Sallusto, F., Geginat, J., Lanzavecchia, A., 2004. Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu. Rev. Immunol. 22, 745–763.

Schwartz, R.H., 1990. A cell culture model for T lymphocyte clonal anergy. Science 248, 1349–1356.

Sharpe, A.H., Freeman, G.J., 2002. The B7-CD28 superfamily. Nat. Rev., Immunol. 2, 116–126.

Shearer, G.M., Payne, S.M., Joseph, L.J., Biddison, W.E., 1984. Functional T lymphocyte immune deficiency in a population of homosexual men who do not exhibit symptoms of acquired immune deficiency syndrome. J. Clin. Invest. 74, 496–506.

Shearer, G.M., Bernstein, D.C., Tung, K.S., Via, C.S., Redfield, R., Salahuddin, S.Z., Gallo, R.C., 1986. A model for the selective loss of major histocompatibility complex self-restricted T cell immune responses during the development of acquired immune deficiency syndrome (AIDS). J. Immunol. 137, 2514–2521.

Sieg, S.F., Bazdar, D.A., Harding, C.V., Lederman, M.M., 2001. Differential expression of interleukin-2 and gamma interferon in human immunodeficiency virus disease. J. Virol. 75, 9983–9985.

Smith, B.L., Krushelnycky, B.W., Mochly-Rosen, D., Berg, P., 1996. The HIV nef protein associates with protein kinase C theta. J. Biol. Chem. 271, 16753–16757.

Sun, Y., Schmitz, J.E., Acierno, P.M., Santra, S., Subbramanian, R.A., Barouch, D.H., Gorgone, D.A., Lifton, M.A., Beaudry, K.R., Manson, K., Philippon, V., Xu, L., Maecker, H.T., Mascola, J.R., Panicali, D., Nabel, G.J., Letvin, N.L., 2005. Dysfunction of simian immunodeficiency virus/simian human immunodeficiency virus-induced IL-2 expression by central memory CD4+ T lymphocytes. J. Immunol. 174, 4753–4760.

Terpstra, F.G., Al, B.J., Roos, M.T., de Wolf, F., Goudsmit, J., Schellekens, P.T., Miedema, F., 1989. Longitudinal study of leukocyte functions in homosexual men seroconverted for HIV: rapid and persistent loss of B cell function after HIV infection. Eur. J. Immunol. 19, 667–673.

Tuosto, L., Marinari, B., Andreotti, M., Federico, M., Piccolella, E., 2003. Vav exchange factor counteracts the HIV-1 Nef-mediated decrease of plasma membrane GM1 and NF-AT activity in T cells. Eur. J. Immunol. 33, 2186–2196.

Veazey, R.S., DeMaria, M., Chalifoux, L.V., Shvetz, D.E., Pauley, D.R., Knight, H.L., Rosenzweig, M., Johnson, R.P., Desrosiers, R.C., Lackner, A.A., 1998. Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. Science 280, 427–431.

Wei, P., Garber, M.E., Fang, S.M., Fischer, W.H., Jones, K.A., 1998. A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. Cell 92, 451–462.

Yue, F.Y., Kovacs, C.M., Dimayuga, R.C., Parks, P., Ostrowski, M.A., 2004. HIV-1-specific memory CD4+ T cells are phenotypically less mature than cytomegalovirus-specific memory CD4+ T cells. J. Immunol. 172, 2476–2486.